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(71) Applicant *(for all designated States except US)*: PEPTIDE THERAPEUTICS LIMITED [GB/GB]; Peterhouse Technology Park, 100 Fulbourn Road, Cambridge CB1 9PT (GB).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: JOHNSON, Tony [GB/GB]; 10 Brookside Grove, Littleport, Ely, Cambs. CB6 1JN (GB). QUIBELL, Martin [GB/GB]; 23 Fennec Close, Cherry Hinton, Cambs. CB1 4GG (GB). HOWE, Joanne [GB/GB]; 2 Berrylands, Milton Road, Cambs. CB4 1XW (GB).

(74) Agent: DAVIES, Jonathan, Mark; Reddie & Grose, 16 Theobalds Road, London WC1X 8PL (GB).

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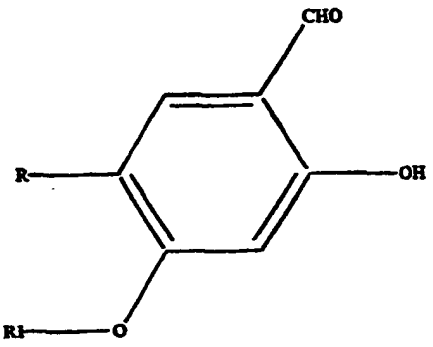
(54) Title: A SOLID-PHASE TECHNOLOGY FOR THE PREPARATION OF COMBINATORIAL LIBRARIES THROUGH AMIDE-BOND ANCHORING

(57) Abstract

The present invention provides the means to suppress epimerisation of the Cα terminal amino acid of a protected peptide sequence during coupling by using the protection moiety shown in (1) which is referred to as a "precursor linker". This moiety has a number of features; the functional group R and the 2-hydroxyl function lie in a para position relative to each other while the ether residue lies in a para position relative to the aldehyde residue. R1 is an electron donating alkyl group. The R group is a moiety that may readily be interconverted between electron-withdrawing and electron donating. This is based on the safety catch principle. The principle, that a stable bond is smoothly converted to a labile one at a convenient point during a synthesis, has been applied in peptide chemistry for the development of linkers and protecting groups. One approach has been to exploit the facile reductive conversion of a sulphoxide to sulphide. This approach when applied to the precursor linker (1) provides the functional protection moieties which are referred to as "linker compounds".



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A Solid-Phase Technology for the Preparation of Combinatorial Libraries Through Amide-bond Anchoring

Introduction

Combinatorial chemistry techniques, which are methods for the parallel preparation of many molecules compared to traditional single serial techniques, have the potential to play a pivotal role in the design and development of drug-like molecules. International Application No. WO 97/40065 describes a combinatorial library technology which has been developed as a tool to accelerate the development of inhibitors of proteolytic enzymes. A protease is screened against a large addressable library of potential protease substrates, swiftly providing an assay for proteolytic activity based upon internally quenched fluorescence. Along with the establishment of a sensitive assay, a wealth of substrate structure-activity data is gathered which may be used in the design of an inhibitor.

International Application No. WO 98/17628 describes a novel solid-phase methodology allowing flexible variation of the N and C termini of a discrete target compound, or a combinatorial approach leading to parallel preparation of many analogues of a target compound. This approach allows for great flexibility in the primary sequence assembly of many chemical classes of compound libraries. However, a potential limitation to International Application No. WO 98/17628 may be encountered when considering a further level of complexity in library synthesis - the absolute chiral integrity of all chiral centres in the target molecules. Under certain circumstances the Cα terminal amino acid of an assembled sequence can undergo epimerisation with the resulting loss of chiral integrity.

Here, a chemical method for the C-terminal activation and coupling of solid phase bound protected peptide and peptidomimetic sequences, with no Cα epimerisation, is described. The method allows the synthesis of combinatorial

libraries where full flexibility of the choice of required residues is needed for the target compounds.

It will readily be appreciated by those skilled in the art that a general solid phase combinatorial route to the desired molecule would not be restricted to the development of peptides but would include non-peptides and also those containing peptidomimetics. Moreover, any molecule useful for any type of interaction e.g. receptor agonists, antagonists for which these molecules exhibit activity may be developed in a combinatorial manner. Here, a novel solid-phase methodology is described allowing the flexible variation of required residues, and allowing a combinatorial approach leading to parallel preparation of many molecules.

Background Chemistry - The Current Problem

Solid phase based syntheses utilise a cross-linked polymer (a resin support) which is functionalised with a chemically reactive unit (a linker). A functional group (carboxylic acid, amine, hydroxyl, sulphydryl etc) from an initial intermediate of the final desired compound is reversibly and covalently attached to the resin through the linker. Sequential chemical transformations of this now resin-bound intermediate to the final compound are then performed. At each stage, excess and spent reagents are removed from the growing resin-bound product by simple filtration and washing - this being the overriding factor providing expedient synthesis compared to solution based synthesis. As a final step, the fully assembled product is released from the solid support by cleavage of the covalent bond between the linker and product functional group.

Traditional solid phase peptide synthesis utilises a linker derivatised resin support to which the Cα carboxyl of the C-terminal residue is covalently attached. The desired sequence is sequentially assembled (using individual elements at each stage to give a single final product or using mixtures of elements at each stage to give a mixture or 'library' of final products). Then the product is released into

solution by cleavage of the C-terminal residue - linker bond. This provides the free C-terminal carboxylic acid. To provide alternative C-terminal functionalities different linkers have been developed. However virtually all linkers described to date release a functional group (carboxylic acid, amine, primary amide, hydroxyl, sulphydryl etc) present in the final product. Thus an obvious problem arises if the desired compound is devoid of one of the above functionalities. For example peptidyl acyloxymethyl ketones, a potent class of inhibitor of the cysteinyl protease *Der p* I, a major allergen of the house dust mite contain no obvious functional group by which a linker can attach an intermediate to a resin. Therefore current solid phase techniques cannot prepare many types of potential drug candidates as single discrete compounds let alone defined libraries of analogues.

A Novel Solid-Phase Based Solution

i) Strategy

The only functional element that is always required to be present in the target molecule is a single secondary amide group. Thus, the attachment of initial intermediates through the conserved secondary amide group to a resin support provides a unique route to any class of linear compounds. Following subsequent solid phase assembly of the desired compound/s, the covalent bond between the linker and now tertiary amide is cleaved to regenerate the conserved secondary amide. During the sequential chemical transformations leading to the final secondary amide product, one has two options. Coupling reactions (the addition of a new chemical moiety providing a part of the final product) may be performed using single building blocks, leading to a single final product. Alternatively, each coupling stage may be performed using chemical mixtures, providing a combinatorial library of final products in which both the N and C terminal residues have been varied. This latter route greatly expands the number and range of drug-like molecules that may be accessed in an overall drug discovery programme.

ii)Chemistry

The vast majority of solid phase syntheses described over the last decade use side-chain functional group protection which is removed by acidolytic cleavage together with Nα-protection removed by base. The wide range of commercially available building blocks is thus based upon this Scheme. A popular strategy in solid phase synthesis is, as a final synthetic step, the concomitant removal of side-chain protection along with product-linker cleavage. Thus, many linkers described in the literature are cleaved from the product by acidolytic treatment. A further desirable feature of a linker is the ability to readily derivatise with a wide range of reagents. An ideal linker should therefore encompass all of the above properties.

There are a number of backbone amide protecting groups which generate amides upon acidolytic treatment described in the literature. Johnson, Quibell and Sheppard have described the development of a backbone amide protection system. This system (not a linker in its own right) was designed to protect the backbone amide of a peptide (previously attached to the resin through a C-terminal residue-linker moiety) during synthesis. Following completion of peptide assembly, the group was removed as a final step along with side-chain deprotection and peptide-linker cleavage by trifluoroacetic acid (TFA). It was found that the use of a 2-hydroxyl rather than a 2-methoxy group allowed the subsequent acylation to be performed with a wide range of reagents, through an acyl transfer mechanism. In contrast, the 2-methoxy derivatised system cannot undergo the acyl transfer reaction and was found to have a very limited applicability.

The group of Barany have recently described a backbone amide linker. This linker does not contain the acyl transfer option during acylation and is therefore not of general applicability.

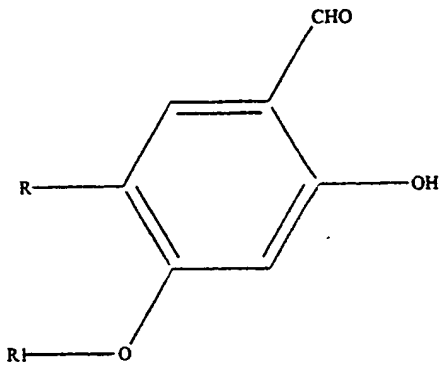
International Application No. WO 98/17628 describes a novel solid-phase methodology which contains an acyl transfer element along with the correct chemical properties of the backbone amide linker making the system compatible with a wide body of commercially available reagents. The linker provides the necessary chemistry to achieve the general goal, being the flexible combinatorial preparation of many libraries of different classes of drug-like molecules having both N and C terminal residues variable simultaneously.

However, this linker has the limitation that under certain circumstances a Cα terminal amino acid can undergo epimerisation with the resulting loss of chiral integrity. Classically, when reactions are performed in a particular sequence where an amino acid residue is activated in the absence of urethane protection, loss of chiral integrity may occur. An example of this is the preparation of backbone cyclised peptides. Here, the C-terminal amino acid residue of a peptide sequence is activated, without urethane protection, facilitating closure to a cyclic peptide and

may give significant epimersiation of the Cα of the activated amino acid residue. This problem severely restricts the fully flexible synthesis of cyclic peptides, with full chiral integrity, to a method of preparation which relies on the activation of a glycine (achiral), or proline (good chiral stability) respectively.

Summary of the Invention

The present invention provides the means to suppress epimerisation of the Cα terminal amino acid of a protected peptide sequence during coupling by using the protection moiety shown in (1) which is referred to as a "precursor linker" by virtue of having the aldehyde group.



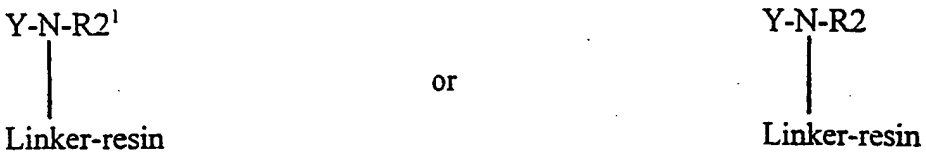
(1)

This moiety has a number of features; the functional group R and the 2-hydroxyl function lie in a para position relative to each other while the ether residue lies in a para position relative to the aldehyde residue. R1 is an electron donating alkyl group.

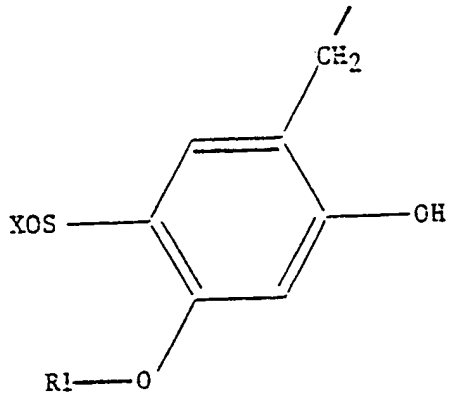
The R group is a moiety that may readily be interconverted between electron-

withdrawing and electron donating. This is based on the safety catch principle. The principle, that a stable bond is smoothly converted to a labile one at a convenient point during a synthesis, has been applied in peptide chemistry for the development of linkers and protecting groups. One approach has been to exploit the facile reductive conversion of a sulphoxide to sulphide. This approach when applied to the precursor linker (1) provides the functional protection moieties which are referred to as "linker compounds".

In a second aspect the present invention provides a resin linked compound of the general formula:



wherein the linker moiety has the general formula (B)



(B)

wherein

X is $(CH_2)_nR3$

where R3 is an -CO- group for attachment to the terminal NH group of the solid phase through a standard bond eg carboxyl amide;

R1 is methyl or another such suitable alkyl known in the art;

n is between 2 and 12, preferably 4;

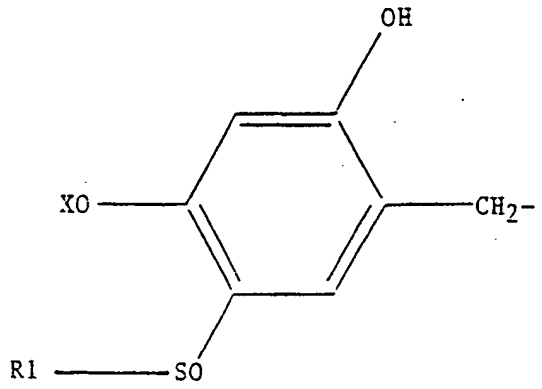
Y is H or an Nα functional group protective moiety such as Fmoc;

R2 is a variable residue; and

R2' is an intermediate form of R2 which is subsequently chemically transformed to give the desired R2.

In a third aspect the present invention provides an alternative presentation of

the linker whereby the attachment to the solid phase via X is through the ether residue of (B), as shown in (C).



(C)

wherein

X is $(CH_2)_nR3$,

where R3 is an -CO- group for attachment to the terminal NH group of the solid phase through a standard bond eg carboxyl amide;

R1 is methyl or another such suitable alkyl known in the art; and

n is between 2 and 12, preferably 4.

The invention provides a methodology for producing the compounds above as well as providing for the compounds which are the products of the said methods.

It is of particular advantage that compounds of the invention may be utilised for the preparation of a combinatorial library of compounds of general formula R_4-CO-NH-R_2, wherein R_4 and R_2 are both variable residues.

Definition: As used herein the term "X is $(CH_2)_nR3$" is to be understood to encompass embodiments in which $(CH_2)_n$ is selected from: (a) a linear alkyl group;

(b) a branched alkyl group; and (c) a non-aromatic ring system which may optionally be attached to a linear or branched alkyl group.

Example Use of the Novel Technology

(A) Preparation of an Acid Derivative Precursor Linker of General Formula (1)

The compound was prepared as detailed in Scheme 1. In that reaction scheme, the preparative steps were as follows:

Scheme 1 Steps

(i) (a) $CuSO_4$ / NH_4SCN (b) H_2SO_4.

(ii) Isobutylene / DCM / MeTf.

(iii) NaOH / Dioxane / H_2O.

(iv) DMSO.

(v) CH_3I / Cs_2CO_3 / MEK.

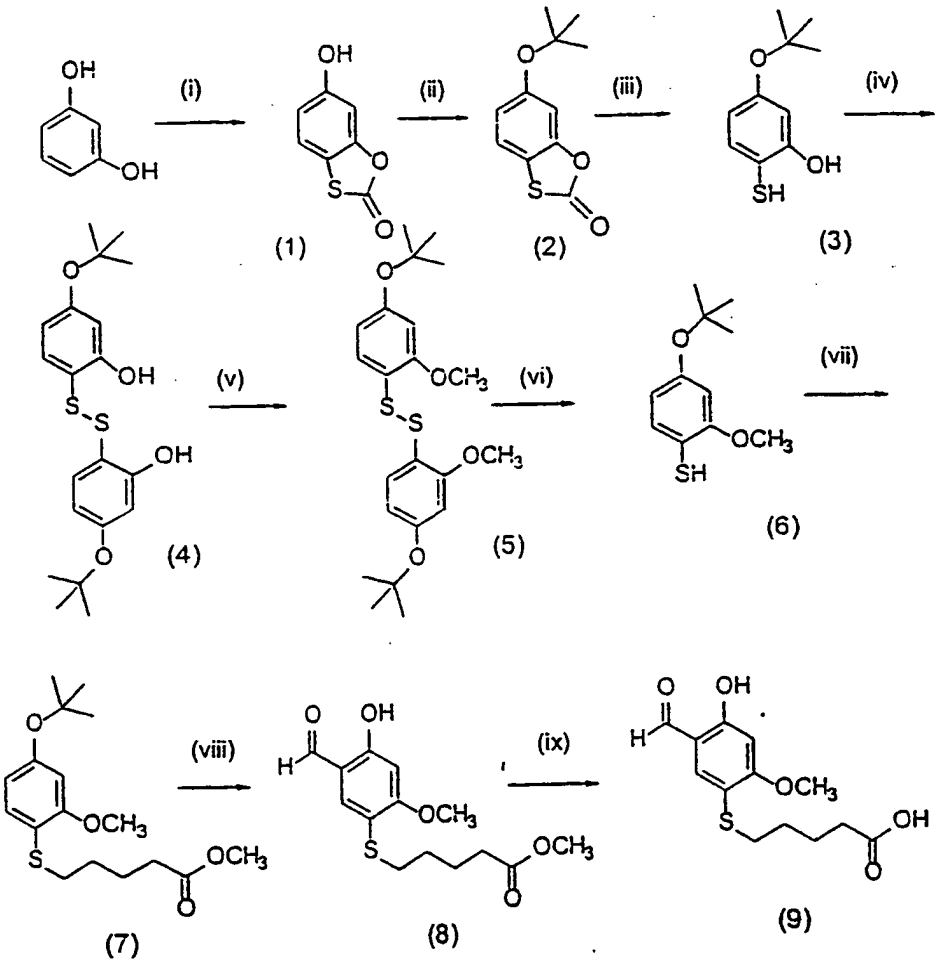
(vi) $NaBH_4$ / EtOH / H_2O.

(vii) Methyl bromovalerate / Cs_2CO_3 / MEK.

(viii) $POCl_3$ / DMF / Dichloroethane.

(ix) LiOH / EtOH / H_2O.

Scheme 1


OH
(i)
OH
OH
O
S
O
(1)
(ii)
O
O
S
O
(2)
(iii)
O
OH
SH
(3)
(iv)
O
OH
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(4)
(v)
O
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H
OCH3
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(8)
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OH
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OCH3
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OH
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(9)

General Experimental procedures

Analytical HPLC was performed using a Phenomenex Jupiter C4 reversed phase column (250 x 4.6 mm id). Solvent system used: solvent A: 0.1% aqueous TFA. Solvent B: 90% acetonitrile, 10% solvent A. Analytical gradient used 10% solvent B, to 90% solvent B over 27 min. Mass spectra were recorded using a Fisons VG single platform spectrometer in either positive ES (electrospray) or APCI (Atmospheric Pressure Chemical Ionisation) mode. 400MHz NMR were acquired at the University of Cambridge NMR service. Chemical shifts are reported in parts per million and were referenced to residual solvent peaks within deuterated solvents. DEPT (distortionless enhancement through polarization transfer) analysis is noted by (0); C<u>H</u> or C<u>H</u>$_3$ (positive) and (1); <u>C</u> or C<u>H</u>$_2$ (negative) signals. Analytical thin-layer chromatography (TLC) was conducted on prelayered silica gel Plates. Visualisation of plates was accomplished using a 254 nm UV light (for chromophores). Flash chromatography was conducted upon Kieselgel 60, 230-400 mesh and was run under a slight positive pressure. Solvents used were either reagent or HPLC grade. Reactions were carried out at ambient temperature under nitrogen unless otherwise noted. Solvent mixtures are expressed as volume: volume ratios. All starting materials were commercially available unless otherwise stated.

Preparation of 6-Hydroxy-2H-1,3-benzoxathiol-2-one. (i)

Resorcinol (0.1mmol, 11g) and copper sulphate (0.2mmol, 31.8g) were dissolved in water (250mL) with vigorous stirring. Ammonium thiocyanate (0.4mmol, 31g) in water (50mL) was added in one portion, the solution turned from blue to black and was left to stir at room temperature for 2 hours. The resultant white suspension was filtered through celite; and washed with a further 50mL of water. The resultant filtrate was stirred vigorously with the addition of sodium carbonate (0.5mmol, 5.3g) in water (50mL) in one portion. After 10min the precipitate formed was filtered and dissolved in concentrated HCl (140mL) and water (260mL) with careful heating for 1 hour at 100^{0}C. The solution was filtered whilst hot with product precipitation occurred upon cooling to room temperature. The resulting precipitate was filtered and washed with cold water and recrystalised from boiling water. Excess water was removed *in vacuo* to yield a white solid m.p. 159^{0}C.

^{1}H NMR (D$_6$ DMSO) δ 10.01 (1H, brs, ArO<u>H</u>), 7.47 (1H, d, *J* 8.6 Hz, Ar<u>H</u>), 6.83 (1H, d, *J* 2.2 Hz, Ar<u>H</u>), 6.74 (1H, dd, *J* 2.2, 6 Hz, Ar<u>H</u>)

^{13}C NMR (D_6 DMSO) δ 169.86 (1), 157.58 (1), 148.37 (1), 123.65 (0), 113.10 (0), 110.64 (1), 99.58 (0).

HPLC retention 10.01min.

APCI-MS (positive mode) found 169 (MH^+), calculated $C_7H_4O_3S$ (167.98)

Preparation of 6-(*tert*-butoxy)-2H-1,3-benzoxathiol-2-one. (2)

A suspension of 6-hydroxy-2H-1,3-benzoxathiol-2-one (12mmol, 2g), in DCM (25mL), the solution was cooled to -30^0C. Isobutylene was condensed into the pressure tube (5mL) followed by the addition of methyl triflate (1.9mmol, 450 L). The sealed tubes were allowed to come to room temperature, and stirred for 3 hours at which point the suspension became colourless. The reaction was cooled to -30^0C and the reaction quenched with N-methyl morpholine (12mmol, 1.11mL), and allowed to come to room temperature. Water (50mL) was added and the aqueous solution was extracted with ethyl acetate (2 x 50mL). The organics were washed with saturated brine (2 x 50mL), 0.5M $KHSO_4$ (2 x 50mL) and finally water (2 x 50mL). The organics were dried over $MgSO_4$, filtered and solvent removed *in vacuo*, yielding 2.3g (92% yield) of a pale yellow gum.

^1H NMR ($CDCl_3$) δ 7.24 (1H, d, *J* 8Hz, ArH), 6.94 (1H, d, *J* 2Hz, ArH), 6.88 (1H, dd, *J* 2, 8.5Hz, ArH), 1.34 (9H, s, $C(CH_3)_3$.

^{13}C NMR ($CDCl_3$) δ 169.51 (1), 155.36 (1), 148.26 (1), 122.06 (0), 122.46 (0), 116.80 (1), 108.32 (0), 79.994, (1) 28.72 (0).

HPLC retention 18.35min.

APCI-MS (positive mode)

Preparation of 5-(*tert*-butoxy)-2-sulfonyl phenol (3).

6-(*tert*-butoxy)-2H-1,3-benzoxathiol-2-one (2) (11mmol, 2.49g) was dissolved in dioxane (105mmol, 9.60mL) with vigorous stirring at 5^0C. A 2N solution of NaOH (10.56mL) was added in a dropwise manor over 10 minutes with vigorous stirring. Cooling was maintained for 10 minutes further then the solution was allowed to warm to room temperature for 1 hour. Dioxane was removed *in vacuo*, the remaining slurry was taken up in water (10mL) and washed with *tert*-butyl ether (20mL). The aqueous layer was carefully acidified with 1N HCl to pH 3 and extracted with ethyl acetate (2 x 20mL). The resultant organic layer was dried over magnesium sulphate, filtered and solvent removed *in vacuo* to yield 1.52g, (71%) of a pale yellow solid.

^{1}H NMR (D_6 DMSO) δ 7.06 (1H, d, *J* 8.3Hz, ArH), 6.46 (1H, d, *J* 2.5Hz, ArH), 6.34 (1H, dd, *J* 2.5, 8.3Hz, ArH), 3.30 (1H, s, OH or SH), 1.23 (9H, s, $C(CH_3)_3$.

^{13}C NMR (D_6 DMSO) δ 153.52, (1), 129.150, (0) 115.19, (0) 112.07, (1) 110.69, (0) 77.67, (1), 28.49, (0).

HPLC retention: 15.62min.

Preparation of 5-(*tert*-butoxy)-2-{[-(*tert*-butoxy)-2-hydroxyphenyl] disulfanyl} phenol. (4).

5-(*tert*-butoxy)-2-sulfonyl phenol (3) (7.7mmol, 1.52g) was dissolved in the minimum amount of DMSO (10mL) with vigorous stirring for 15 hours. The reaction was quenched with water (200mL) and extracted with tert-butyl ether (2 x 50mL). The organics were dried over $MgSO_4$ and solvents removed *in vacuo* yielding 1.41g (93%) of a pale yellow gum, which was stored under Argon.

^{1}H NMR (D_6 DMSO) δ 7.25 (2H, d, *J* 8.5 Hz, 2 x ArH), 6.45 (2H, d, *J* 2.5 Hz, 2 x ArH), 6.41 (2H, dd, *J* 2.5, 8.5 Hz, 2 x ArH), 1.27 (18H, s, 2 x $C(CH_3)_3$)

^{13}C NMR (D_6 DMSO) δ 153.52, (1), 129.150, (0) 115.19, (0) 112.07, (1) 110.69, (0) 77.67, (1), 28.49, (0).

ES-MS (positive mode): Found 395 (MH^+): Calculated $C_{20}H_{26}O_4S_2$ (394.13)

Preparation of di [4-(*tert*-butoxy)-2-methoxyphenyl] disulfide. (5).

5-(*tert*-butoxy)-2-{[-(*tert*-butoxy)-2-hydroxyphenyl] disulfanyl} phenol. (4) (3.11mmol, 1.22g), methyl iodide (9.35mmol, 3mL) and caesium carbonate (12mmol, 3.9g) were suspended in methyl-ethyl ketone (12.5mL) and stirred under nitrogen for 15 hours. The suspension was filtered through celite, washed with ethyl acetate (2 x 10mL) and solvent removed *in vacuo* yielding 1.2g (90%) of a pale red oil.

^{1}H NMR (D_6 DMSO) δ 7.37 (2H, d, *J* 8.5 Hz, 2 x ArH), 6.52-6.62 (4H, m, 4 x ArH), 3.76 (6H, s, 2 x OCH_3), 1.30 (18H, s, 2 x $OC(CH_3)_3$.

^{13}C NMR (D_6 DMSO) δ 157.39,(1), 156.59, (1), 129.75, (0), 117.11, (0), 115.32, (0), 107.08, (0), 78.57, (1), 55.81, (0), 28.46, (0).

HPLC retention: 25.09min.

ES-MS (positive mode); found 423, (MH^+), calculated $C_{22}H_{30}O_4S_2$ 422.59.

Preparation of 4-(*tert*-butoxy)-2-methoxyphenyl hydrosulfide. (6).

Di [4-(*tert*-butoxy)-2-methoxyphenyl] disulfide, (5) (2.1mmol, 900mg) was dissolved in ethanol (4mL) and cooled to 0^0C in a NaCl-ice bath. A solution of sodium borohydride (4mmol, 0.15g) in water (5mL) was added dropwise over 15 minutes, allowed to come to room temperature over 1 hour and stirred at room temperature for 6 hours. Solvent was removed *in vacuo*; the resultant residue was redissolved in water (10mL) and acidified to pH 5 with 1N HCl. The acidified solution was washed with ethyl acetate (2 x 10mL), solvent removed *in vacuo* yielding 800mg (88%) of a pale yellow solid.

^{1}H NMR (D_6 DMSO) δ 7.20 (1H, d, *J* 8.3Hz, ArH), 6.56 (1H, d, *J* 2.3Hz, ArH), 6.50 (1H, dd, *J* 2.3, 8.3Hz ArH), 3.78 (3H, s, OCH_3), 1.28 (9H, s, $OC(CH_3)_3$.

^{13}C NMR (D_6 DMSO) δ 155.18, (1), 153.87, (1), 128.87, (0) 116.10, (0), 114.06, (1), 107.72, (0), 78.06, (1), 55.73, (0), 28.47, (0).

HPLC retention: 18.48 min.

ES-MS (positive mode); found 213 (MH^+). Calcuated $C_{11}H_{16}O_9S$ (212).

Preparation of methyl 5-{[4-(*tert*-butoxy)-2-methoxyphenyl] sulfanyl} pentanoate. (7).

4-(*tert*-butoxy)-2-methoxyphenyl hydrosulfide, (6) (2.3mmol, 500mg), methyl-5-bromovalerate (3.6mmol, 1mL) and caesium carbonate (16.2mmol, 5.2g) were suspended in methyl-ethyl ketone (5mL) and refluxed under nitrogen at 85^0C for 4 hours. The suspension was allowed to cool, filtered through celite and solvent removed *in vacuo* yielding a yellow oil.

^{1}H NMR (D_6 DMSO) δ 7.13 (1H, d, *J* 8.2 Hz, ArH), 6.54 – 6.57 (2H, m, 2 x ArH), 3.76 (3H, s, OCH_3), 3.58 (3H, s, $COOCH_3$), 2.81 (2H, t, *J* 7.1Hz, SCH_2), 2.50 (2H, m, $COCH_2$), 1.81 (2H, dt, *J* 6.0, 7.1Hz, CH_2CH_2), 1.63 (2H, dt, *J* 7.1, 7.8 Hz, CH_2CH_2), 1.31 (9H, s, $C(CH_3)_3$)

^{13}C NMR DMSO (D_6 DMSO)

δ 173.05,(1), 157.46, (1), 154.84, (1) 129.47, (0), 117.58, (1), 115.45, (0) 107.27, (0), 78.16, (1) 55.13, (0), 51.13, (0), 34.45, (1), 32.21, (1), 31.44, (1), 27.813, (0), 23. 00, (1).

HPLC retention: 20.07 minutes.

ES-MS (positive mode); found 327, (MH^+), calculated $C_{17}H_{26}O_4S$, 326.45.

Preparation of methyl 5-[(5-formyl-4-hydroxy-2-methoxyphenyl)sulfanyl] pentanoate. (8)

Methyl 5-{[4-(*tert*-butoxy)-2-methoxyphenyl] sulfanyl} pentanoate (7) (0.6mmol, 200mg) was dissolved in dichloroethane (1.02mmol, 124 L) and DMF (0.9mmol, 100 L) at 0^0C. To this vigorously stirred solution was added $POCl_3$ (1.02mmol, 124 L) in a dropwise manor over 15 minutes. After addition, the solution was maintained at 0^0C for a further 60 minutes and room temperature for 16 hours. The reaction was quenched by the slow addition of ice (100mL) over 1 hour, extraction of the aqueous solution occurred via the addition of DCM (2 x 50mL). The organic phase was washed with saturated brine (2 x 50mL) and dried over $MgSO_4$, filtered and solvent removed *in vacuo* to yield 150 mg (84% crude, 1:4 ratio of non-formylated: formylated product) of a dark yellow gum, which was purified by column chromatography.(eluant; ethyl acetate/ hexane)

^{1}H NMR (D_6 DMSO) δ 9.83 (1H, s, COH), 7.13 (1H, d, *J* 8.2 Hz, ArH), 6.54 – 6.57 (1H, m, ArH), 3.76 (3H, s, OCH_3), 3.58 (3H, s, $COOCH_3$), 2.93 (2H, t, *J* 7.1Hz, SCH_2), 2.50 (2H, m, $COCH_2$), 1.83 (2H, dt, *J* 6.0, 7.1Hz, CH_2CH_2), 1.63 (2H, dt, *J* 7.1, 7.8 Hz, CH_2CH_2).

^{13}C NMR DMSO (D_6 DMSO)

δ 190.01 (0)173.05,(1), 157.46, (1), 151.66, (0) 129.47, (0), 117.58, (1), 115.45, (0) 107.27, (0), 78.16, (1) 55.13, (0), 34.45, (1), 32.21, (1), 31.44, (1), 27.81, (0).

HPLC retention: 16.7 minutes.

ES-MS (positive mode): Found 299 (MH^+). Calculated $C_{14}H_{18}O_5S$ (298).

Preparation of 5-[(5-formyl-4-hydroxy-2-methoxyphenyl) sulfanyl] pentanoic acid. (9)

Methyl 5-[(5-formyl-4-hydroxy-2-methoxyphenyl)sulfanyl] pentanoate (8) (0.8mmol, 235mg) was dissolved in a solution of LiOH (9mmol, 210mg) in Methanol: water (3:1, 5mL). The solution was stirred at room temperature for 15 hours then acidified to pH4 with 1N HCl, extracted into ethyl acetate (2 x 40mL),

dried over $MgSO_4$. The solvent was removed *in vacuo* yielding 200mg (88%) of a pale yellow oil.

^1H NMR (D_6 DMSO) δ 9.84 (1H, s, COH), 7.13 (1H, d, *J* 8.2 Hz, ArH), 6.55 – 6.57 (1H, m, ArH), 3.76 (3H, s, OCH_3), 2.93 (2H, t, *J* 7.1Hz, SCH_2), 2.50 (2H, m, $COCH_2$), 1.83 (2H, dt, *J* 6.0, 7.1Hz, CH_2CH_2), 1.63 (2H, dt, *J* 7.1, 7.8 Hz, CH_2CH_2),

^{13}C NMR DMSO (D_6 DMSO)

δ 190.01 (0), 173.05 (1), 157.46 (1), 151.66 (0) 129.47 (0), 117.58 (1), 115.45, (0) 107.27 (0), 78.16 (1) 55.13 (0), 51.13 (0), 34.45 (1), 31.44 (1).

HPLC retention: 13.93 minutes.

ES-MS (positive mode). Found: 285 (MH^+). Calculated $C_{13}H_{16}O_5S$ (284).

(B) Preparation of an alternative Acid Derivative Precursor Linker of the General Formula 1

The compound was prepared as detailed in Scheme 2. In that reaction Scheme the preparative steps were as follows:

Scheme 2 Steps

(i) (a) $CuSO_4$ / NH_4SCN (b) H_2O

(ii) Isobutylene / CH_2Cl_2 / MeTf

(iii) NaOH / Dioxane / H_2O

(iv) DMSO

(v) Methyl bromovalerate / Cs_2CO_3 / MEK

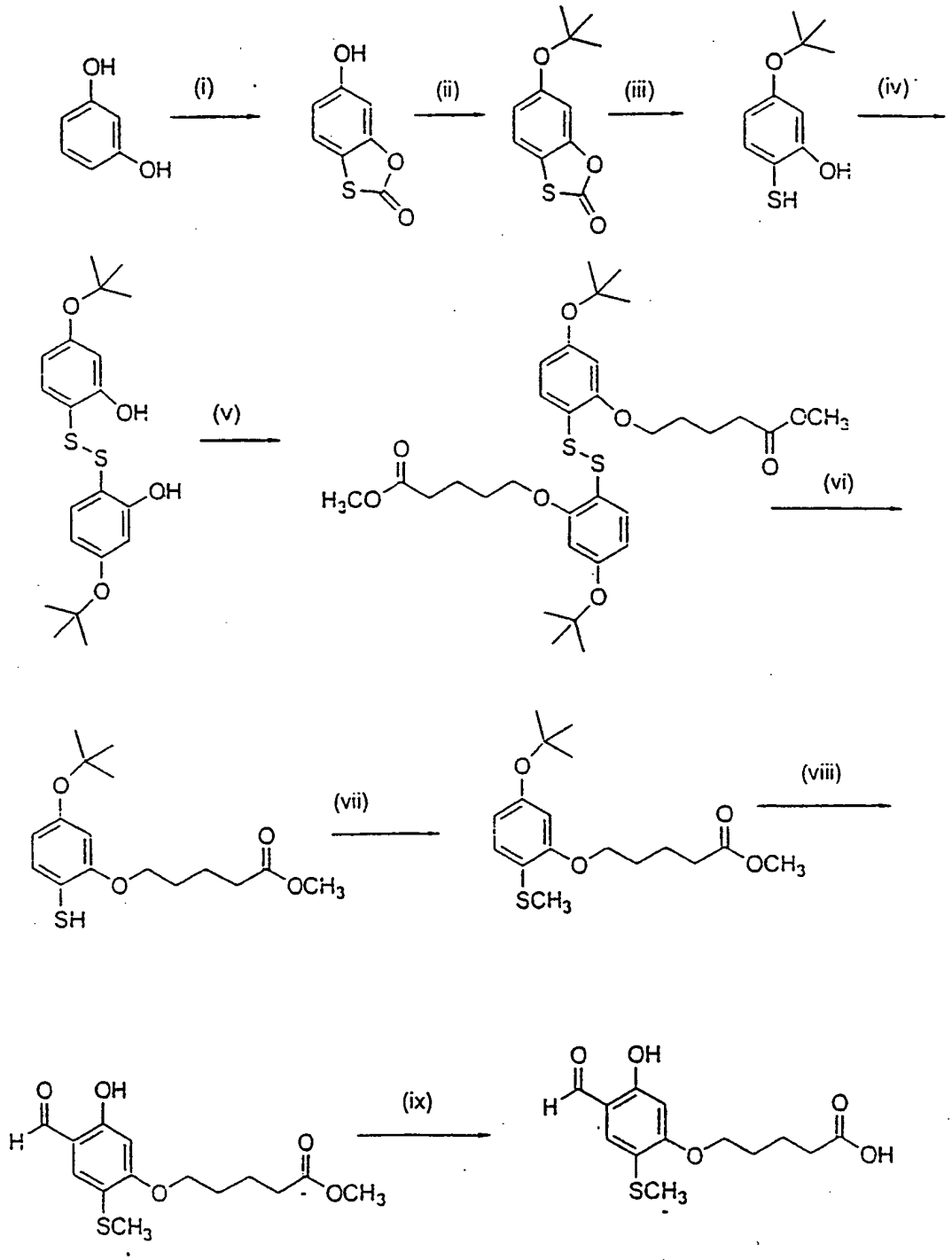
(vi) $NaBH_4$ / ethanol /H_2O

(vii) CH_3I / $CsCO_3$ / MEK

(viii) $POCl_3$ / DMF / Dichloroethane

(ix) LiOH / ethanol / H_2O

Scheme 2

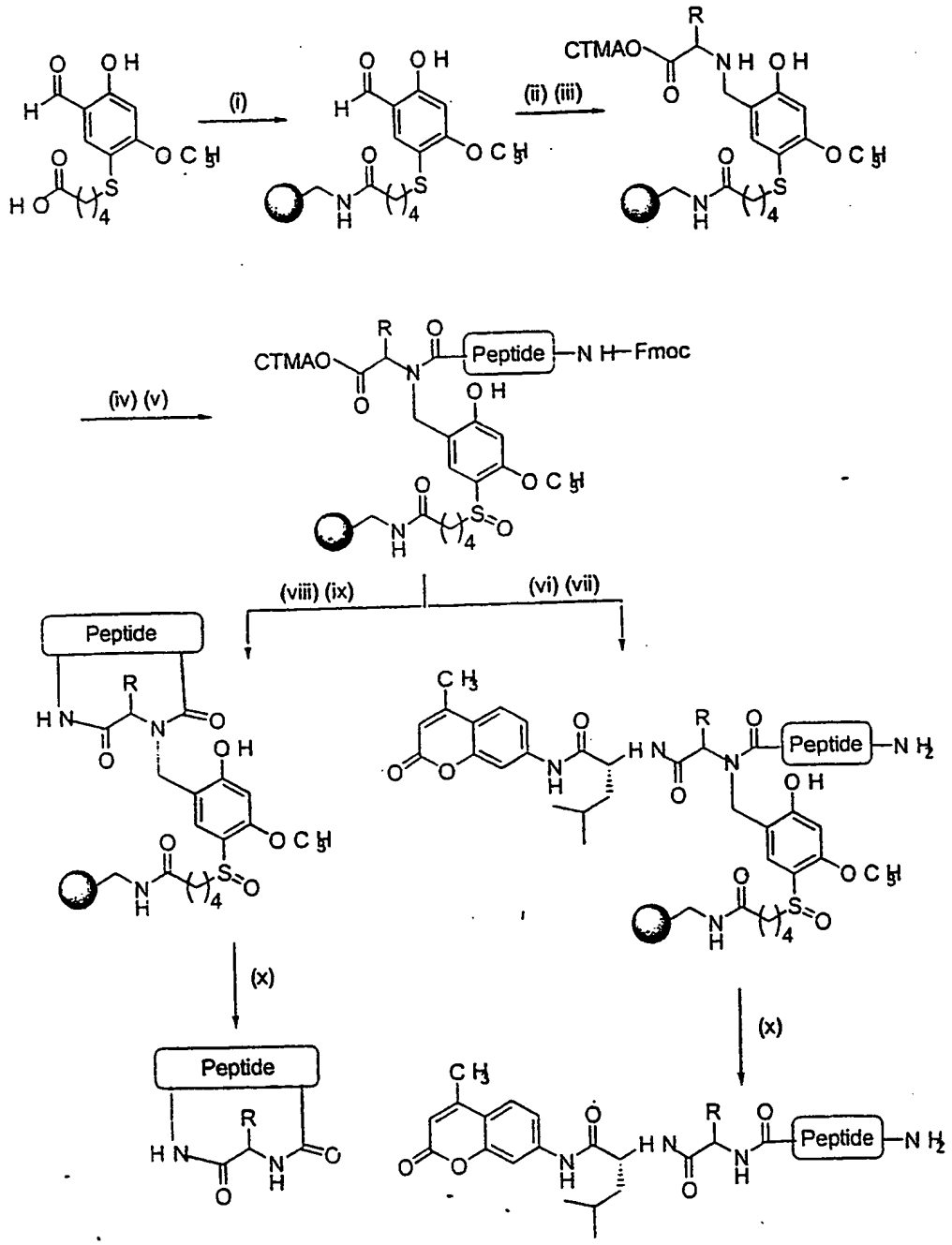


(C) Example

Libraries of compounds have been synthesised using the novel solid phase combinatorial chemistry of the present invention.

Example 1

Scheme 3



Example 1 Steps for Scheme 3

(i) Novasyn TG resin / BOP / HOBt / NMM / DMF

(ii) H-AA-O-CTMA / TOMF

(iii) (a) $NaCNBH_4$ / THF /AcOH / H_20 (b) Fmoc-Cl / DIEA / DMF

(iv) Standard Fmoc-polyamide synthesis

(v) MCPBA / DCM

(vi) 1% TFA / DCM

(vii) H-Leu-AMC /BOP / HOBt / NMO

(viii) 1%TFA / DCM

(ix) BOP / HOBt / DIEA / DMF

(x) TFA / DMS / NH_4I

Scheme 3

General Procedures for use of the Sulfoxide Handle.

Incorporation of Handle (9) onto NovaSyn TG amino resin. (10)

5-[(5-formyl-4-hydroxy-2-methoxyphenyl) sulfanyl] pentanoic acid. (9) (2.8mmol, 800mg), BOP (2.8mmol, 1.06g), and HOBt (4.2mmol, 642mg) were combined and dissolved in DMF (10mL), N-methyl morpholine (4.2mmol, 390μL) was added, and after 5 minutes preactivation, this solution was added to NovaSyn TG amino resin (0.24mmol g^{-1}, 4g). The reaction was allowed to proceed for 8 hours. The resultant resin-handle complex was washed with DMF (5 x 2 min), CH_2Cl_2 (5 x 2 min), MeOH (3 x 2 min) and TBDME (5 x 2 min). The resin was dried initially under a positive nitrogen pressure and then *in vacuo*.

Incorporation of first residue onto BAL-TG-resin via reductive amination.(11).

Linker-resin complex (10) was allowed to eqilibrate in trimethylorthoformate (15mL) containing H-L-Leu-CTMA (28mmol, 4.62g). The reaction is allowed to proceed in a capped syringe for 5 hours. The resultant resin-complex was washed with dry THF (5 x 2 min) and dried under a positive pressure of nitrogen. The resin was then suspended in dry THF/ acetic acid/water (90:5:5, v/v/v, 20mL) containing sodium cyanoborohydride (14mmol, 882mg) for 14 hours. The resultant resin conjugate was washed with DMF/water (9:1, v/v, 20mL x 9), MeOH (20mL x 9) and tert-butyl ether (30mL x 1) allowing the solvent to percolate through the resin bed for 30 seconds. The resin conjugate was dried under a positive nitrogen pressure and *in vacuo* for 2 hours.

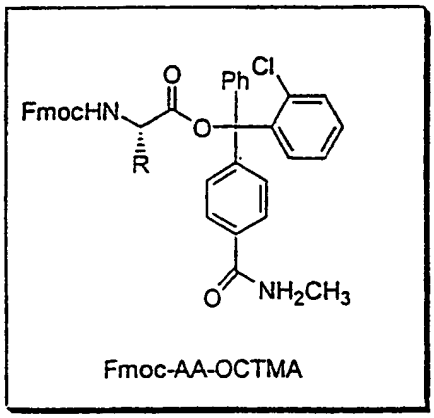
Oxidation of the resin bound peptide-linker conjugate.

The resin-linker–AA-OCTMA complex was initially reated with Fmoc-Cl and DIEA to protect the the secondary amine nitrogen prior to oxidation of the sulfide. The nitrogen dried resin conjugate (500mg) was suspended in a solution of MCPBA (250mg in 25mL DCM). The reaction was allowed to proceed in a capped syringe for 5 hours. The resultant resin-complex was washed with DCM (2 x 20mL), DMF (2 x 20mL), methanol (2 x 20mL) and finally tert-butyl ether (2 x 20mL). The resin conjugate was dried under a positive nitrogen pressure and finally in a vacuum desicator overnight.

Acylation of resin conjugate (11) via symmetrical anhydrides. (A general case)

Fmoc-amino acid (20eq excess to resin loading) is dissolved / suspended in dichloromethane (5mL / mmol amino acid) with stirring and ice cooling in a 50mL Falcon tube. If the amino acid appears insoluble , then DMF (500µL) is added to aid dissolution. Diisopropylcarbodiimide(10eq) in DCM (1mL) is added over a few minutes, and the mixture stirred at 0^0C for 30 minutes. Resin conjugate (11) was added to the anhydride solution, sealed and left to react for the appropriate time. The fully acylated resin cojugate is filtered and washed with DMF (5 x 50mL), methanol (5 x 50mL) and tert-butyl ether (2 x 50mL).

Solid phase peptide chemistry was carried out using standard solid phase Fmoc-AA-Opfp / HOBt couplings. The C-terminal protecting group chosen was the in house developed Fmoc-AA-OCTMA derivatives. These Fmoc amino acid derivatives are stable throughout Fmoc-polyamide peptide synthesis, and can be removed using weak solutions of TFA in conjunction with the appropriate scavengers (TFA / TES).



Fmoc-AA-OCTMA

Removal of the CTMA protecting group.

Peptide-resin conjugate (100mg) was suspended in a solution of 2%TFA / 1%TES in DCM (10mL) for 15 minute x 2. The resin was filtered, washed with DMF (5 x 10mL), methanol (5 x 10mL) and *tert* butyl ether (5 x 10mL) and dried under a positive nitrogen pressure.

Cyclization to give cyclo –(AA_1-AA_2-AA_3-AA_4-AA_5-AA_6)– :

Peptide-resin (50mg, 0.24mmol / g, was treated with suspended in 1ml of a solution of DMF containing BOP (16mg, 0.036mmol), HOBt (6mg, 0.036mmol) and NMM (4μL, 0.036mmol). The resin was aggitated for 24 hours, removal of reagents occurred via filtration, followed by washing with DMF (2 x 2mL), methanol (2 x 2mL) and tert-butyl ether (2 x 2mL). The resin was dried under a positive pressure of nitrogen prior to cleavage.

Coupling of H-AA-AMC to resin bound peptide.

Peptide-resin (50mg, 0.24mmol / g, was treated with suspended in 1ml of a solution of DMF containing BOP (16mg, 0.036mmol), HOBt (6mg, 0.036mmol) and NMM (4μL, 0.036mmol) and H-AA-AMC (0.036mmol). The resin was aggitated for 2 hours, removal of reagents occurred via filtration, followed by washing with DMF (2 x 2mL), methanol (2 x 2mL) and tert-butyl ether (2 x 2mL). The resin was dried under a positive pressure of nitrogen prior to cleavage.

Cleavage from the sulfoxide linker-resin conjugate.

Peptide-resin conjugate (50mg, 0.24mmol g^{-1} substitution) was suspended in TFA / TES / DMS / H_2O (90/1/1/8) containing 1 mol % of NH_4I. The suspension was aggitated at room temperature for 2 hours. The resin was filtered, the resultant filtrate was sparged down under a stream of nitrogen and the peptide precipitated via the slow addition of cold *tert* butyl ether, spun down via centrifugation and air dried prior to HPLC analysis.

Data for compounds prepared utilising resin bound sulfoxide linkage agent.

Linear peptide sequence prepared via Fmoc polyamide synthesis:

Compound of general formula cyclo –(AA_1-AA_2-AA_3-AA_4-AA_5-AA_6)-

H-Leu-Tyr-Leu-Ser-Gln-Leu-OH, $C_{35}H_{37}N_7O_{10}$; calculated 735.89, found 736.5 (MH^+). HPLC retention 12.78 minutes.

Cyclo (-Leu-Tyr-Leu-Ser-Gln-Leu-) ; calculated 717.89, found 719.8 (MH^+). HPLC retention 14.13 minutes

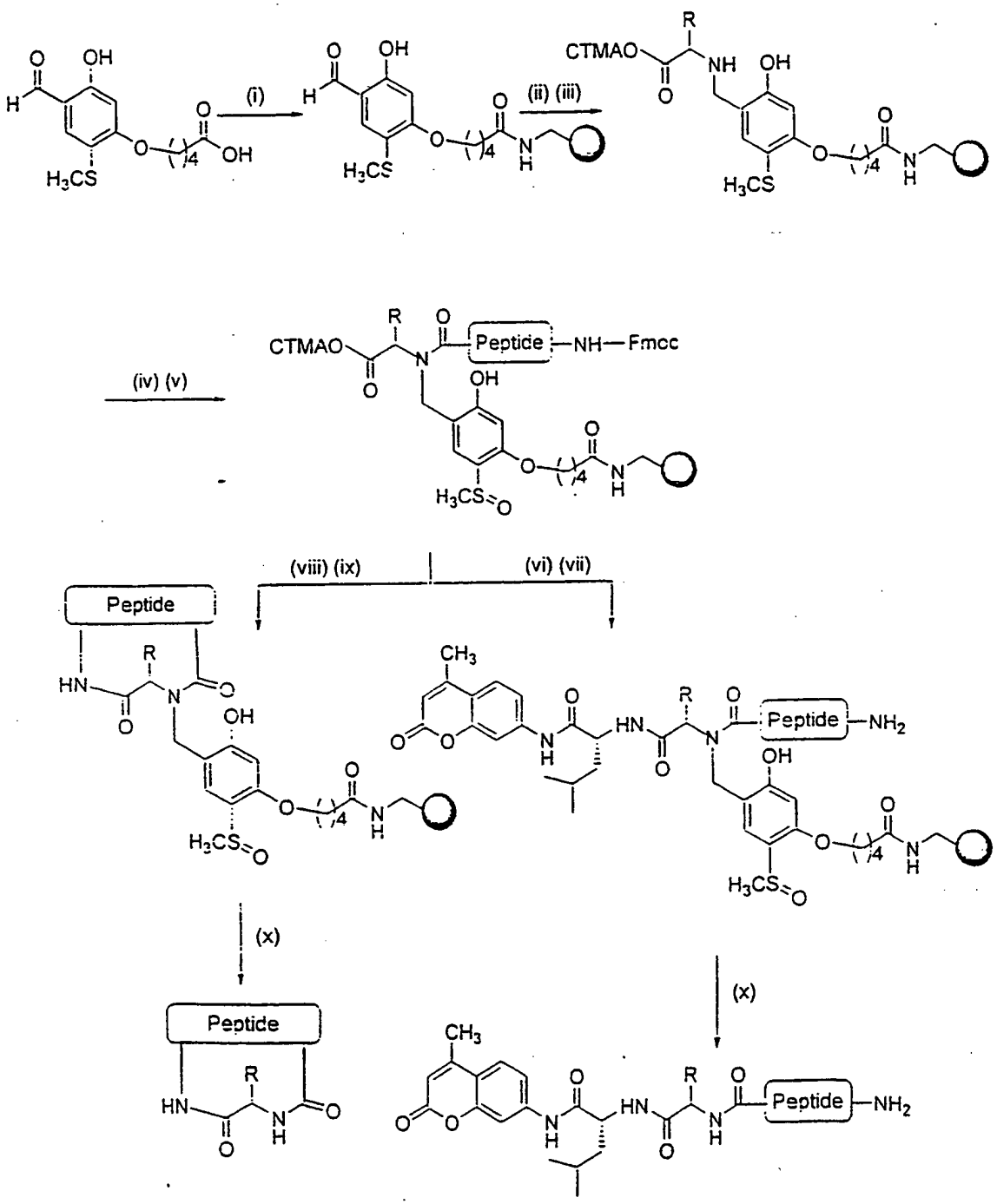
Compound of the general formula X-AA_1-AA_2-AA_3-AA_4-AMC.

Fmoc-Ser-Gln-Leu-OH, $C_{29}H_{36}N_4O_8$; calculated 568.68, found 569.5 (MH^+), 590.9 (MNa^+). HPLC retention 21.03 minutes.

Ac-Ser-Gln-Leu-Leu-AMC, $C_{32}H_{46}O_9N_6$; calculated 658.93, found 659.8 (MH^+), 680.9 (MNa^+). HPLC retention 13.09 minutes.

Example 2

Scheme 4



Example 2 Steps Scheme 4

(i) Novasyn TG resin / BOP / HOBt / NMM / DMF

(ii) H-AA-O-CTMA / TOMF

(iii) (a) $NaCNBH_4$ / THF /AcOH / H_2O (b) Fmoc-Cl / DIEA / DMF

(iv) Standard Fmoc-polyamide synthesis

(v) MCPBA / DCM

(vi) 1% TFA / DCM

(vii) H-Leu-AMC /BOP / HOBt / NMO

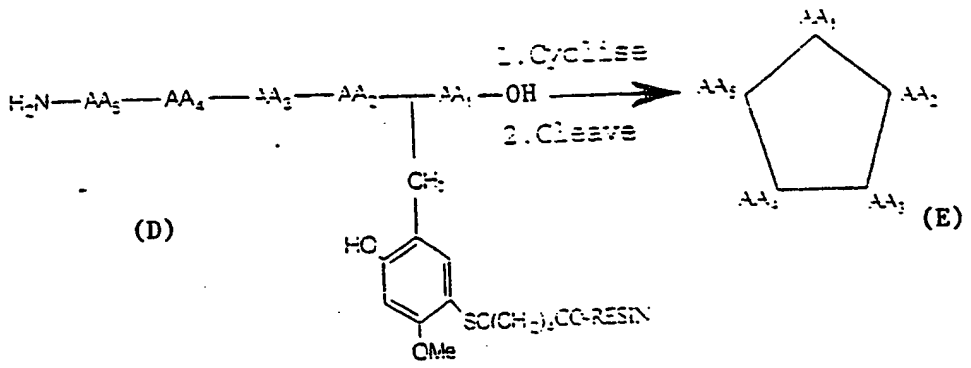
(viii) 1%TFA / DCM

(ix) BOP / HOBt / DIEA / DMF

(x) TFA / DMS / NHLI

Example 3

Combinatorial libraries of peptidyl cyclic compounds which can be cyclised from linear compounds of general formula (D) and cleaved to provide cyclic compounds of the general formula (E) in which AA_1-AA_5 are independently combinatorially variable.



It is a particular advantage of the use of this linker that the class of compound (D) can utilise any available residue, be it a peptide, peptidomimetic or other, as during the synthesis the chiral integrity of the Cα is protected. Therefore, the need to include specialised residues in this position, such as proline or glycine, which cannot easily be epimerised in the reaction, is eliminated.

Thus according to a further aspect of the invention there are provided libraries of compounds and individual compounds per se of the formula (D) whether attached to the linker or in cleaved form together with libraries and individual

compounds per se of the formula (E).

To a person skilled in the art it will be apparent that the use of this technology will enable cyclic compounds with a variable number of residues present including but not limited to AA_1-AA_4, AA_1-AA_5, and AA_1-AA_6.

Example 4

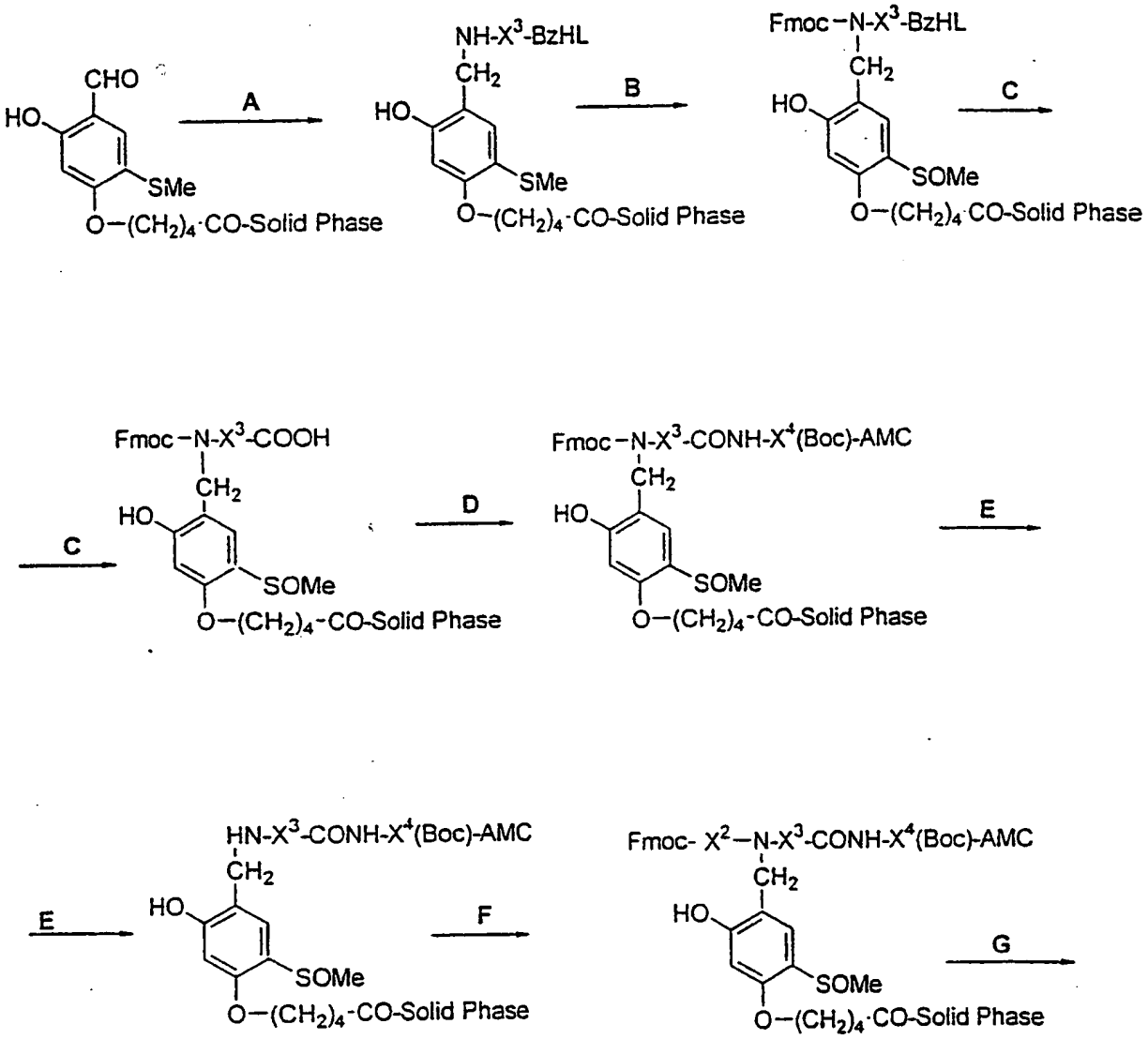
The present invention can be used to produce combinatorial libraries useful for designing appropriate peptide-based substrates and inhibitors for proteases (and other enzymes).

A known class of compounds having three variable amino acid residues X of general formula Ac-X-X-X-Asp-aminomethylcoumarin have been proposed for investigating protease specificities of interleukin-1β converting enzyme (ICE) - ref: Rans *et al*, Chem. & Biol., 1997, Vol. 4, No. 2. A scissile bond between Asp and AMC can be cleaved to release the fluorogenic AMC group. The present invention allows for the production of a general library class of compounds -X-X-X-X-AMC which have four variable amino acid residues X. Such compounds are referred to hereafter as "4X-AMC". The compounds can be synthesised according to the present invention and libraries of the compounds can be used to rapidly and accurately assess enzymatic specificity. In the following description, Scheme 5 provides a generic synthetic route to 4X-AMC compounds, and Scheme 6 provides a specific example of the synthesis of the compound Ac-Tyr-Leu-Leu-Lys-AMC. It is to be noted that the precursor compound prior to step A corresponds to the product of step (ix) of scheme 2 wherein R=SMe and the product is resin bound. Also the product of step B corresponds to the intermediate of claim 1 wherein R_2^1 is the variable residue precursor and Y=Fmoc.

Furthermore, those skilled in the art can readily appreciate that the compound prior to step A could be chosen to have the -SOMe rather than the -SMe substituent. However, due to the interconvertability of these moieties the reductive amination of step A would reduce -SOMe to -SMe followed by re-oxidation to -SOMe in step B.

Example 4(a)

Scheme 5



Scheme for Synthesis of 4X-AMC

A 1. NH_2-X^3-BzHL and
2. Reductive Amination

B. 1. Fmoc-Cl. DIEA. DCM
2. m-chloroperbenzoic acid

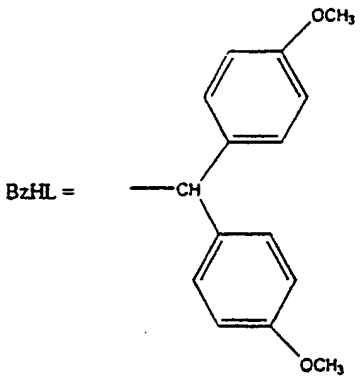
C. 1. 1% TFA. DCM

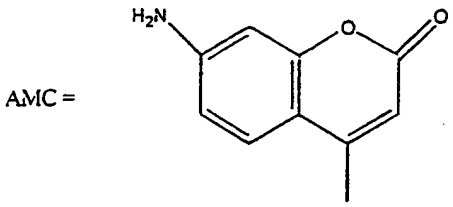
D. 1. NH_2-X^1(Boc)-AMC
2. BOP, HOBt, NMM

E. 1. Piperidine, DMF

F. 1. $(Fmoc\text{-}X^2)_2O$
2. DCM

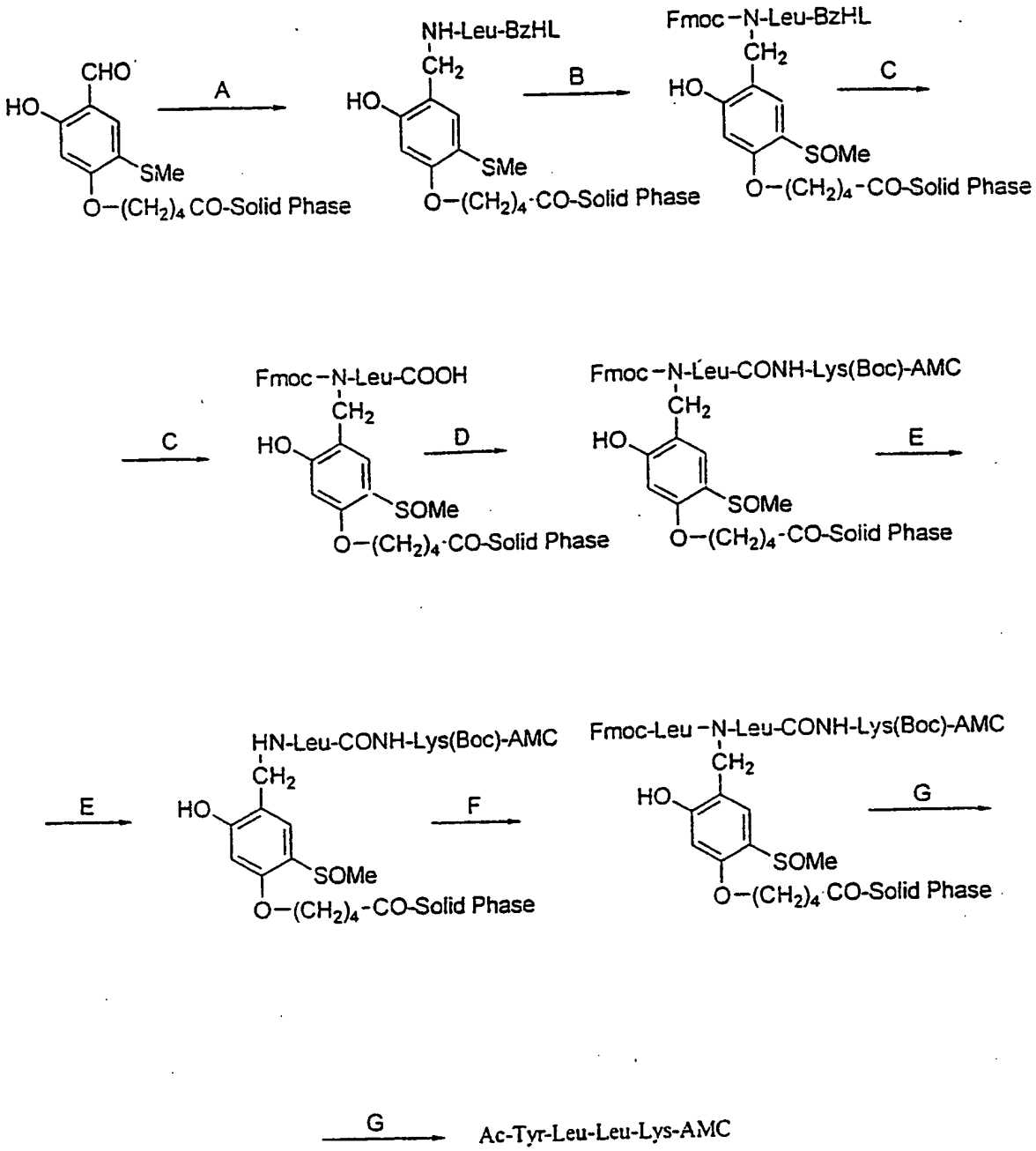
G. 1. Solid-phase synthesis (X^1)
2. Reduction of SOMe to SMe
3. Cleavage





Example 4(b)

Scheme 6



Scheme for Synthesis Ac-Tyr-Leu-Leu-Lys-AMC

A 1. NH_2-Leu-BzHL and
2. Reductive Amination

B. 1. Fmoc-Cl, DIEA, DCM
2. m-chloroperbenzoic acid

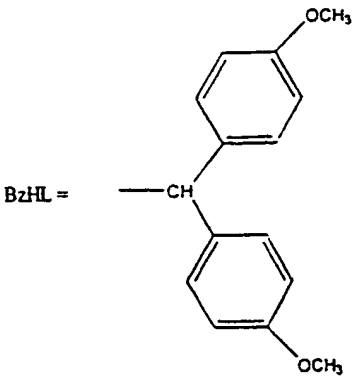
C. 1. 1% TFA, DCM

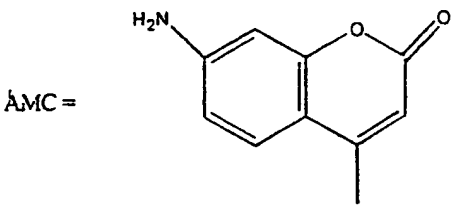
D. 1. NH_2-Lys(Boc)-AMC
2. BOP, HOBt, NMM

E. 1. Piperidine, DMF

F. 1. $(Fmoc\text{-}Leu)_2O$
2. DCM

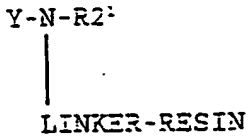
G. 1. Solid-phase synthesis
2. Reduction of SOMe to SMe
3. Cleavage



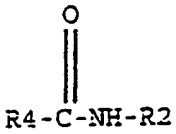


Claims

1. An intermediate compound of general formula



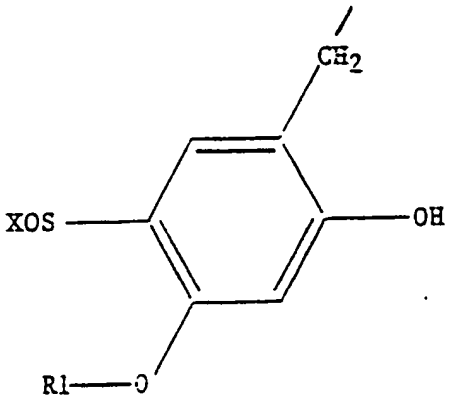
for use in a method of preparation of a compound of general formula (A)



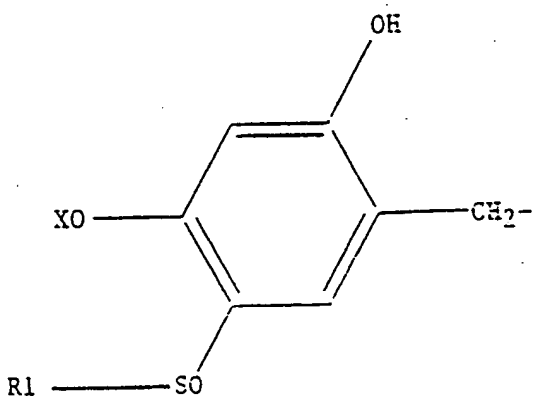
(A)

wherein R4 is a variable residue; and

wherein the linker moiety has the general formula (B) or (C)



(B)



(C)

wherein

X is $(CH_2)_nR3$

where R3 is an -CO- group for attachment to the terminal NH group of the solid phase through a standard bond eg carboxyl amide;

R1 is methyl or another such suitable alkyl known in the art;

n is between 2 and 12, preferably 4;

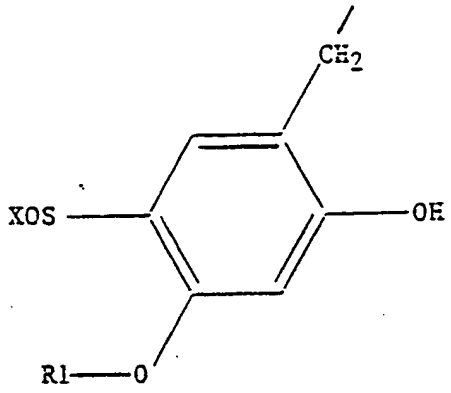
Y is H or an Nα functional group protective moiety such as Fmoc;

R2 is a variable residue; and

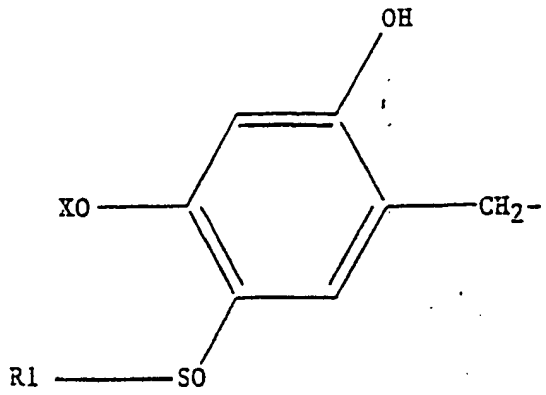
$R2^1$ is an intermediate form of R2 which is subsequently chemically transformed to give the desired R2.

2. An intermediate compound of general formula

wherein the linker moiety has the general formula (B) or (C)



(B)



(C)

wherein

X is $(CH_2)_nR3$

where R3 is an -CO- group for attachment to the terminal NH group of the solid phase through a standard bond eg carboxyl amide;

R1 is methyl or another such suitable alkyl known in the art;

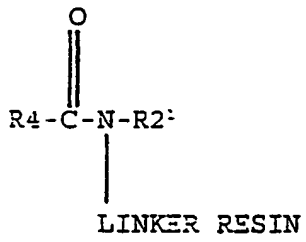
n is between 2 and 12, preferably 4;

Y is H or an Nα functional group protective moiety such as Fmoc;

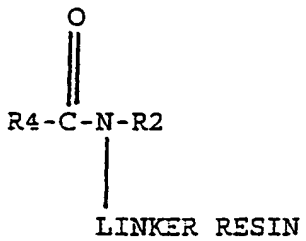
R2 is a variable residue; and

$R2^1$ is an intermediate form of R2 which is subsequently chemically transformed to give the desired R2.

3. An acyl derivative of an intermediate compound according to claim 1 or 2 having the general formula $(F)(F^1)$



(F^1)



(F)

wherein R4 is an amino acid, peptide or peptidomimetic sequence which is combinatorially variable.

4. A method which comprises the steps of Scheme 1, for making a precursor linker compound of formula 1 for use in a method of preparation of a resin-bound intermediate compound according to claim 1 or claim 2.

5. A method which comprises the steps of Scheme 2, for making a precursor linker compound of formula 1 for use in a method of preparation of a resin-bound intermediate compound according to claim 1 or claim 2.

6. A method which comprises the steps of Scheme 3.

7. A method which comprises the steps of Scheme 4.

8. A method which comprises the steps of

i) forming a plurality of intermediate resin linked compounds according to claim 1 or claim 2;

ii) forming from said intermediates a plurality of resin linked compounds having variable residues R4 and R2 or R4 and $R2^1$ therein;

iii) cleaving the compounds from the linkers to produce a plurality of compounds of general formula A; and

iv) optionally transforming $R2^1$ to R2 either before or after cleavage;

whereby the product of step (iii) or (iv) provides a combinatorial library of compounds of formula (A).

9. A method which comprises the steps of Scheme 5, for producing a compound of general formula Ac-X-X-X-X-AMC.

10. A method according to claim 9 which comprises forming a plurality of said compounds Ac-X-X-X-X-AMC having variable residues X, whereby the product of the method is a combinatorial library of said compounds.

11. A resin linked compound which is the product of step (iv)(v) of Scheme 3 or the product of step (iv)(v) of Scheme 4 (optionally having one or both protective groups shown in said Schemes varied to be another protective group).

INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 98/03523

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C07B61/00 C07K1/04 C07C59/74

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07B C07K C07C

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 92 18144 A (LEBL MICHAL ;PATEK MARCEL (US)) 29 October 1992 see page 1, line 5 - line 24 see claim 1 ---	1-3,6-9, 11
A	CALMES M ET AL: "USE OF A NEW SONICALLY CLEAVABLE AND ACIDO-LABILE HANDLE FOR SOLID-PHASE PEPTIDE AMIDE SYNTHESIS" INTERNATIONAL JOURNAL OF PEPTIDE AND PROTEIN RESEARCH, vol. 44, no. 1, 1 July 1994, pages 58-60, XP000451983 see page 58, paragraph 1 - paragraph 2 see schemes 1 and 2 --- -/--	1-3,6-9, 11

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
23 April 1999	07/05/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Held, P

INTERNATIONAL SEARCH REPORT

Inter. nal Application No
PCT/GB 98/03523

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	WO 96 00378 A (AFFYMAX TECH NV ;HOLMES CHRISTOPHER (US)) 4 January 1996 see figures 1,4,5 see claim 1	1-3,6-9, 11
P,A	WO 98 17628 A (JOHNSON TONY ;QUIBELL MARTIN (GB); PEPTIDE THERAPEUTICS LTD (GB)) 30 April 1998 cited in the application see scheme 4 see claim 1	1-3,6-9, 11

INTERNATIONAL SEARCH REPORT

information on patent family members

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